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Antibody Prevalence and Specificity to Group C Rotavirus in Swedish Sera

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Of 160 sera collected from different age groups throughout Sweden, 38% were found to be antibody positive for group C rotavirus. The highest antibody prevalence rate was found in individuals aged 11–30 years (45%). An immunoprecipitation assay revealed that the antibodies were directed against VP2, VP4, VP6, VP7, and NSP2, with VP6 being the most immunogenic protein. Neutralising antibodies against a cultivable porcine group C rotavirus (strain AmC-1/Cowden) were detected in 16/19 individuals at titres from 160 to 5,120. The results indicate that group C rotavirus infections are relatively common in older Swedish children and adults but appear to be less common in children younger than 5 years of age. It is concluded that porcine and human group C rotaviruses share epitopes critical for stimulation of neutralising antibodies. ***J. Med. Virol. 60:210–215, 2000.***

KEY WORDS: atypical rotavirus; gastroenteritis; immune response; radioimmunoprecipitation; neutralisation antibodies

INTRODUCTION

Rotaviruses are established as important causes of acute gastroenteritis in humans and animals. Based on immunological and biochemical characteristics they are subdivided into seven distinct serogroups (A–G) [Saif, 1990]. Groups A–C are human pathogens with group A rotaviruses being the clinically most important, causing an estimated 870,000 deaths/year worldwide [de Zoysa and Feachem, 1985]. With the exception of gastroenteritis outbreaks caused by group B rotavirus in China [Den et al., 1984; Chen et al., 1985], most identified non-group A rotaviruses appear to belong to group C [Espejo et al., 1984; Bridger et al., 1986; Brown et al., 1989; Matsumoto et al., 1989; Penaranda et al., 1989; Caul et al., 1990; Maunula et al., 1992; Jiang et al., 1995]. Apart from the association with acute diarrhoea, group C rotaviruses have also been reported to be associated with extrahepatic biliary atresia [Riepenhoff-Talty et al., 1996].

In contrast to group A rotavirus, the epidemiology and clinical importance of group C rotavirus has not been established. Recent seroepidemiological investigations have revealed seroprevalences of 30–66% from different parts of the world [James et al., 1997; Riepenhoff-Talty et al., 1997], suggesting that group C infections might be more common than previously recognised, but the difficulties in detecting these viruses have precluded proper assessment of their clinical importance.

Group C rotavirus has been propagated in swine testicular (ST) cells [Welter et al., 1991]. The cultivation system has provided insight on the biochemistry of the virus [Nilsson et al., 1993] as well as possibilities of developing diagnostic methods to improve surveillance of these viruses.

A seroepidemiological study of group C rotavirus in Sweden is described. The aim was to establish the prevalence of group C rotavirus infections in Sweden and characterise for the first time the immune response following a group C rotavirus infection.

MATERIALS AND METHODS

Sera

Among a national collection of Swedish sera, 160 sera were collected randomly from eight different age groups with 20 in each group included in the present study.

Cultivation of Group C Rotavirus

ST cells were grown in Eagle's minimal essential medium (EMEM) supplemented with nonessential amino acids, 2 mM L-glutamine, 0.1 M sodium pyruvate, 0.1 M sodium bicarbonate, 0.1 M HEPES, 50 μg/ml Gentamycin, and 10% foetal calf serum as described elsewhere [Welter et al., 1991; Nilsson et al., 1993].

Confluent monolayers of ST cells in roller tubes were rinsed three times with serum-free EMEM before in-

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fection with cultivable porcine group C rotavirus (strain AmC-1/Cowden) [Welter et al., 1991; Svensson, 1992; Nilsson et al., 1993]. Briefly, group C rotavirus was trypsin activated (10 μg/ml, type III, Sigma, St. Louis, MO, USA) for 30 min at 37°C and then inoculated on ST cells. After 1 hr of incubation at 37°C, the inoculum was removed and serum-free EMEM containing 1 μg/ml of trypsin was added. Infection was allowed to continue until a complete cytophatogenic effect was observed at 2–3 days post-infection (p.i.), at which time cells were freeze-thawed twice and cell debris removed by low speed centrifugation.

For immunofluorescence testing, Lab-Tek chambers (Nunc Inc., Naperville, IL) with confluent monolayers of ST cells were infected with trypsin-activated porcine group C rotavirus. After 12–16 hr of infection in serum-free and trypsin-free medium, cells were fixed in cold methanol, dried, and stored at −70°C until use.

Immunofluorescence Assay (IF)

Sera were diluted 1:10 in phosphate-buffered saline (PBS) and incubated on infected and mock-infected ST cell monolayers in Lab-Tek chambers for 30 min at 37°C. After rinsing three times in PBS, FITC-conjugated goat anti-human IgG (Dako, Glostrup, Denmark) diluted 1:40 was added and incubated for 30 min at 37°C. The cells were finally rinsed three times in PBS, mounted and inspected for specific fluorescence. Positive sera were given scores of +, ++, +++, ++++ depending on the brightness of the fluorescence.

Metabolic Labelling and Radioimmunoprecipitation (RIPA)

Metabolic labelling of group C rotavirus-infected cells and immunoprecipitation were performed essentially as described elsewhere [Svensson et al., 1987; Nilsson et al., 1993]. Briefly, ST cells were mock infected or infected at a multiplicity of five with group C rotavirus. At 6 hr p.i., infected and mock-infected cells were starved for 1 hr in methionine-free medium before labelling with 50 μCi ^{35}S-methionine (Amersham, Pharmacia, Uppsala, Sweden) for 1 hr. Cells were then lysed in ice cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 0.5% Trasylol-Aprotinin pH 7.8) and centrifuged for 2 min at 5,000 rpm in an Eppendorf centrifuge to remove cellular debris.

For immunoprecipitation, 50 μl of labelled cell lysate and 10 μl serum were mixed with 450 μl RIPA buffer (10 mM Tris, 150 mM NaCl, 600 mM KCl, 5 mM ethylenediamine tetraacetic acid (EDTA), and 2% Triton X-100 pH 7.8) and incubated overnight at 4°C. Protein A Sepharose (Pharmacia, Uppsala, Sweden) was then added (25 μl) to the mixture and incubation continued on a rocker platform for 2 hr at room temperature. Immune complexes were washed four times in RIPA buffer and twice with 10 mM Tris-HCl, 0.15 M NaCl pH 8.0, before being dissociated by boiling for 5 min in sample buffer (6% SDS, 6% mercaptoethanol, 3 mM EDTA, 20% glycerol, 100 mM Tris HCl, 0.05% bromophenol blue pH 6.8). Proteins were then separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently processed for autoradiography as described elsewhere [Nilsson et al., 1993].

Neutralisation Test (NT)

Fifty microliters of a virus suspension diluted to give 200 peroxidase focus-forming units of virus were mixed with equal volumes of serial twofold dilutions of sera (starting with a serum dilution at 1:40). The mixtures were incubated at 37°C for 1 hr and then inoculated in duplicate on confluent monolayers of ST cells in 96-well plates (Nunc, Denmark). After 1 hr of incubation at 37°C, plates were washed with serum-free EMEM and incubated in serum and trypsin-free EMEM. After 12–16 hr of incubation, cells were fixed in cold methanol and the number of infected cells determined by peroxidase focus reduction test [Svensson, 1992]. Briefly, a rabbit anti-group C rotavirus antiserum, prepared against purified porcine AmC-1/Cowden group C rotavirus, diluted 1:1,000, was added to the wells and incubated for 1 hr at 37°C. After three washes with PBS a peroxidase conjugated goat anti-rabbit IgG antiserum (Bio-Rad, Richmond, CA) diluted 1:3,000 was added and incubated for 1 hr at 37°C. After three more washes in PBS, the reaction was developed at room temperature with 3-amino-ethylcarbazole (Sigma). The number of infected cells were determined and the neutralisation titre was expressed as the reciprocal of the highest dilution giving 60% reduction in the number of peroxidase stained cells as compared with the virus control.

RESULTS

Antibody Prevalence to Group C Rotavirus

In the present study an indirect immunofluorescence test was used to establish the antibody prevalence to group C rotavirus in Sweden. Of the 160 examined sera, 61 (38%) were found to be positive for group C rotavirus antibodies (Fig. 1). Figure 2 illustrates the immunofluorescence testing of a positive and negative sera. Whereas the antibody prevalence was 35% (7/20) in sera from persons ≤ 10 years of age, it reached 45% in the 11–20 and 21–30 age groups and dropped to 40% in the age groups 31–40, 41–50, and 51–60. The lowest antibody prevalences were noted in the two groups containing the oldest individuals, in which only 30% of the sera were positive (Fig. 1). But no single age group had significantly stronger or weaker IF signals than average.

A more detailed examination of the youngest age group revealed that a majority (5/7) of the antibody-positive sera came from individuals between 5 and 10 years of age. It should be noted, however, that only 8 of the 20 sera examined in this group came from individuals older than 5 years, suggesting that infections with group C rotavirus occur predominantly after 5 years of age in Sweden, as only 2/12 of the children younger than 5 years of age were group C antibody positive.

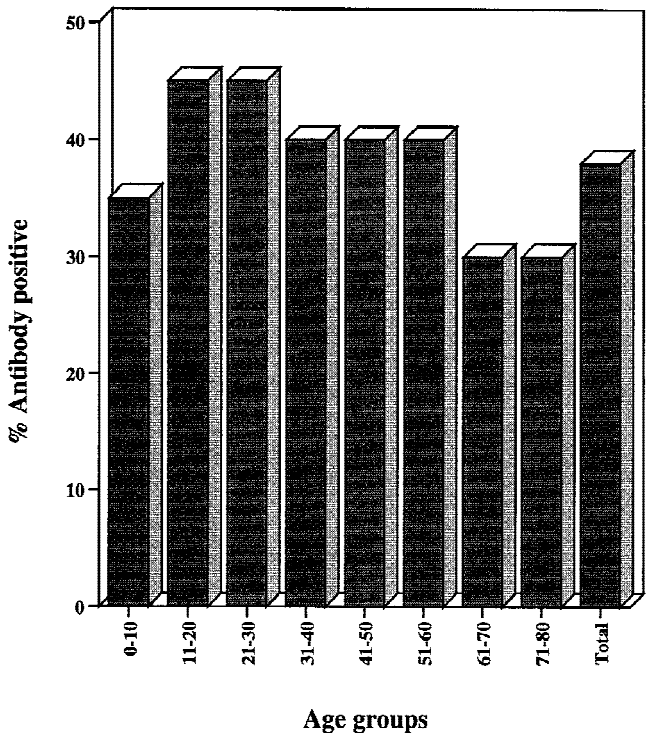

Fig. 1. Antibody prevalence to group C rotavirus in 160 Swedish sera, divided into eight different age groups with 20 sera in each.

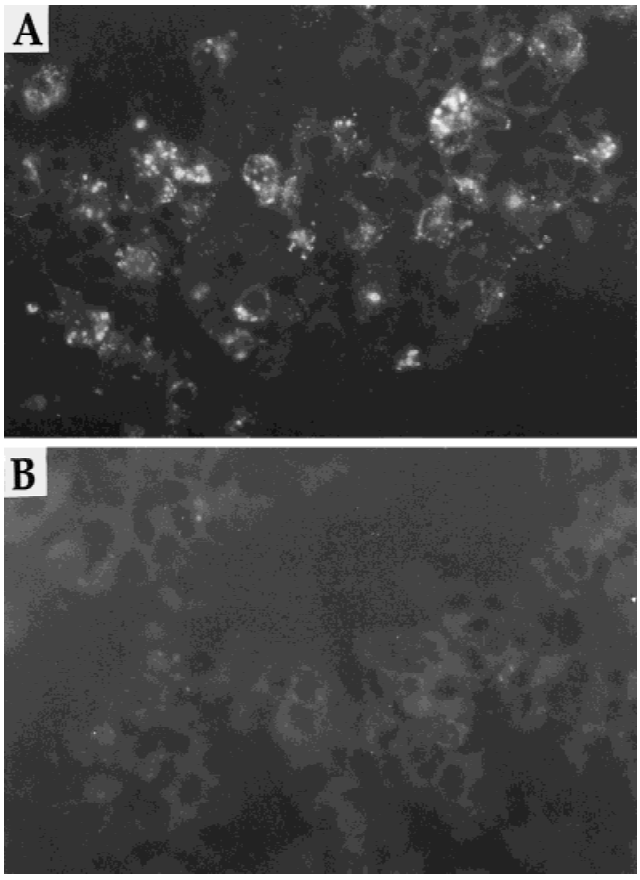

Fig. 2. Demonstration of group C rotavirus-specific IgG antibodies by immunofluorescence. **(A)** A positive serum at a 1:10 dilution with typical cytoplasmatic fluorescence. **(B)** A group C rotavirus-negative serum at 1:10 dilution.

Antibody Responses to Individual Polypeptides of Group C Rotavirus

To obtain an insight into the polypeptide-specific immune responses that develop following a group C rotavirus infection, an immunoprecipitation assay was established essentially as described by Svensson et al. [1987] for group A rotavirus. To illustrate the proteins synthesised by group C rotavirus in ST cells, a figure is presented showing the profile of ^{35}S-labelled polypeptides of the AmC-1/Cowden strain (Fig. 3). The molecular weights of the virus-encoded polypeptides were calculated and the protein profile compared with previous observations [Nilsson et al., 1993].

Twenty sera, 19 group C antibody positive and 1 antibody-negative (#7), were investigated by immunoprecipitation to determine against which polypeptides the immune response was directed. As illustrated in Figure 4, all of the antibody positive sera recognised one or more viral proteins. The immune responses were directed against VP2, VP4, VP6, VP7, and NSP2. Identification of protein-specific immune responses were assisted by identification of VP2, VP4, and VP6 by monoclonal antibodies (Fig. 5) [Nilsson and Svensson, 1995] and by molecular weight comparisons (Fig. 3.) [Nilsson et al., 1993].

The most immunogenic protein was the inner capsid protein VP6, which was recognised by all 19 antibody-positive sera. The outer capsid protein VP7 was recognised by most of the sera, and in comparison with VP2 and VP4, was not only recognised by more sera but appeared also to stimulate stronger antibody response as judged from the intensity of the precipitated bands (Fig. 4). However, it should be stressed that this observation is speculative, as no attempts were made to titrate the sera. In a few sera (# 2, 3, and 5) a faint immunoprecipitated protein was detected slightly below the VP4 protein. As no such protein was observed in uninfected cells (Fig. 4), it might represent a cleavage product of a viral protein, possibly VP4 [Nilsson et al., 1993]. The only nonstructural protein immunoprecipitated was NSP2.

Human Sera Contain Neutralising Antibodies Against Porcine Group C Rotavirus

An aim was to examine the prevalence of neutralising antibodies against group C rotavirus. The rationale was not only to investigate the prevalence of neutralising antibodies per se, but also to gain information about the serotype-specific relationship between porcine and human group C rotaviruses. The occurrence of neutralising group C specific antibodies was examined by using the same sera as used in the immunoprecipitation study (Fig. 4). By a peroxidase focus reduction test, 16/19 group C antibody-positive sera were found

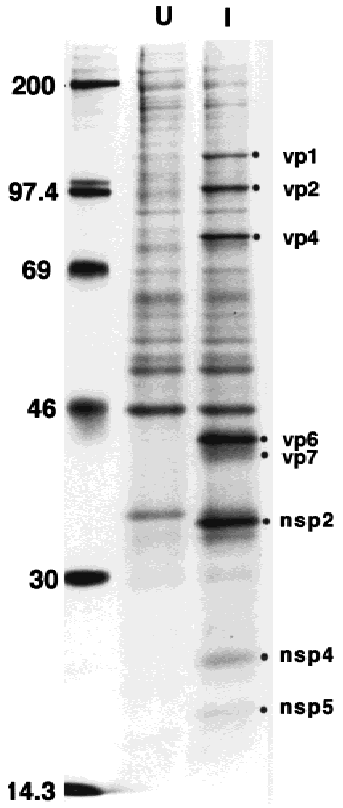

Fig. 3. Group C rotavirus polypeptides analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Uninfected (U) and infected (I) swine testicular (ST) cells were labelled with ^{35}S methionine 7–8 hr postinfection. Viral specific polypeptides are indicated with dots. Molecular weight markers are shown on the left.

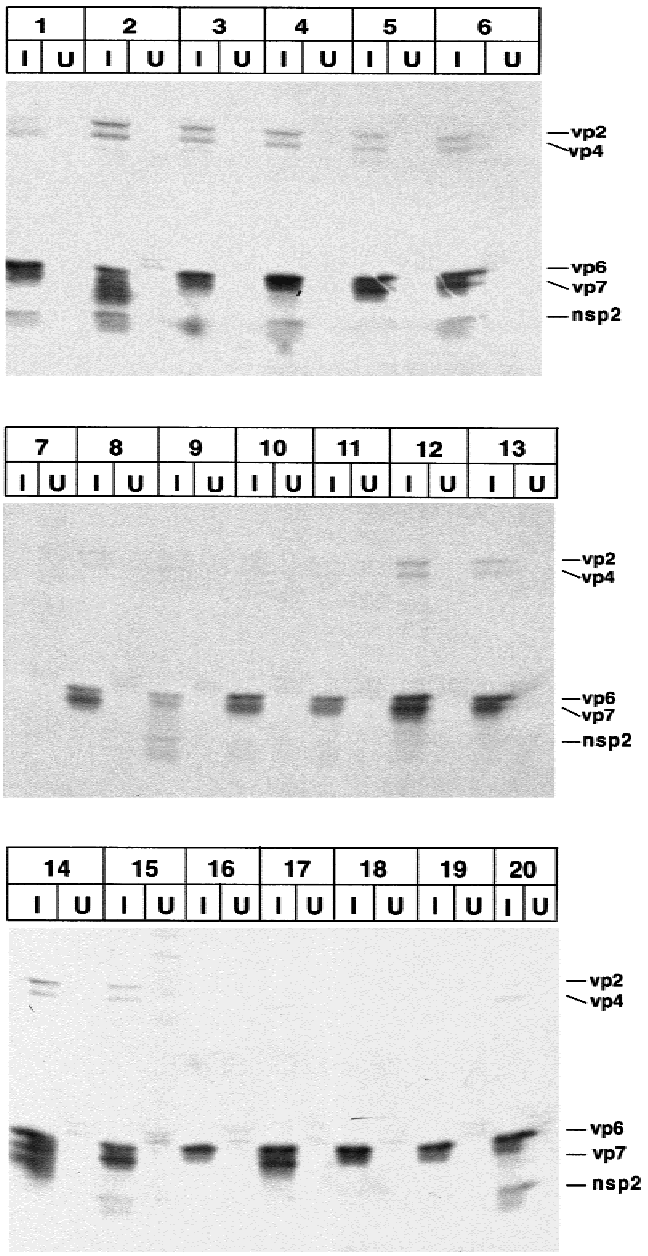

Fig. 4. Immunoprecipitation of porcine group C rotavirus polypeptides by 20 human sera. Infected (I) and Uninfected (U) ST cells.

to contain neutralising antibodies in titres between 160 and 5,120. It is interesting to note that whereas individuals #4, 9, and 18 had no detectable neutralising antibodies, they had group C antibodies by both IF and RIPA. Sera #4 and 18 had not only significant amounts of group C antibodies as judged by RIPA (Fig. 4) and IF, but they also appeared to contain antibodies directed against the VP7 outer capsid protein.

No single age group had significantly higher or lower neutralising antibody titres than average, but high NT titres generally correlated with high IF scores.

DISCUSSION

This seroepidemiological study of group C rotavirus revealed that 38% of the investigated sera, collected randomly throughout Sweden, contained antibodies to group C rotavirus. Recent seroprevalence studies have shown that antibodies to group C rotavirus are present in 30–66% of the adult population in different geographical regions [James et al., 1997; Riepenhoff-Talty et al., 1997], indicating that group C rotavirus infections might be more common than previously recognised by serological surveys [Bridger, 1987; Tsunemitsu et al., 1992a]. There is, however, an apparent discrepancy between serological data and the low or nil detection rates of group C rotavirus reported in humans [von Bonsdorff and Svensson, 1988; Ushijima et al., 1989; Maunula et al., 1992; Jiang et al., 1995; Barnes et al., 1998; Kuzuya et al., 1998]. A possible explanation for this discrepancy could be that searching for etiological agents of acute gastroenteritis has been focused mainly on examining specimens from infants or young children, not necessarily the primary targets for group C rotavirus. In fact, most of the group C viruses detected have been identified in older children and adults [von Bonsdorff and Svensson, 1988; Brown et al., 1989; Matsumoto et al., 1989; Maunula et al., 1992; Kuzuya et al., 1998]. Seroepidemiological data from the present study and previous studies [Es-

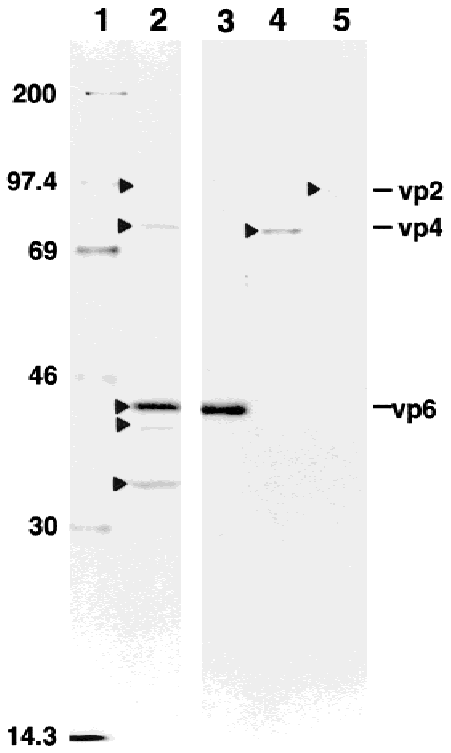

Fig. 5. Identification of group C-specific polypeptides by monoclonal antibodies. **Lane 1**, molecular weight markers; **lane 2**, AmC-1/Cowden polypeptides immunoprecipitated with a human sera; **lane 3**, VP6 immunoprecipitated with a VP6-specific monoclonal antibody (8G5); **lane 4**, VP4 immuno-precipitated with a VP4-specific monoclonal antibody (1A5); **lane 5**, VP2 immunoprecipitated a with VP2-specific monoclonal antibody (2A9).

pejo et al., 1984; Bridger et al., 1986; Tsunemitsu et al., 1992a; James et al., 1997] illustrate differences in prevalences between age groups, with lower seropositivity in children than adults and progressive increases in seropositivity with increasing age [James et al., 1997]. The seroepidemiological data and the detection profiles both suggest that group C rotavirus has an epidemiology distinct from group A rotavirus, which typically infects children before 3 years of age and where virtually all children have seroconverted by the end of the third year of life.

To our knowledge there is no available information regarding the protein-specific immune responses that develop following a group C rotavirus infection. One aim of this study was therefore to uncover the immunogenicity of group C rotavirus proteins using a radioimmunoprecipitation method used previously for group A rotavirus analysis [Svensson et al., 1987; Richardson et al., 1993; Colomina et al., 1998].

Similarly to group A rotavirus [Svensson et al., 1987; Richardson et al., 1993; Colomina et al., 1998], the inner capsid protein VP6 was shown to be the most immunodominant protein. Furthermore, VP2 was found to be significantly immunogenic and antibodies against VP2 were detected in more than 50% of the group C antibody-positive sera. The VP2 observations thus appear to be similar to those observed previously for group A rotavirus [Svensson et al., 1987; Richardson et al., 1993; Colomina et al., 1998]. Most sera immunoprecipitated the outer capsid glycoprotein VP7 and to a lesser extent the hemagglutinin VP4. The immune response to VP7 was surprisingly stronger than the corresponding VP4 response, an observation different from group A rotavirus, in which VP4 has been reported to be more immunogenic than VP7 [Svensson et al., 1987; Richardson et al., 1993; Ward et al., 1993]. A possible explanation for the weak immune response to VP4 could be that VP4 of group C rotavirus displays a smaller portion of conserved epitopes shared between porcine and human strains. A less likely explanation could be that VP4 is more sensitive to proteolytic digestion than VP4 of group A rotavirus, resulting in cleavage of VP4 either during cell lysis or during antibody incubation. The fact that a few sera recognised a viral protein smaller than intact VP4 supports this hypothesis. However, this hypothesis is contradicted by the fact that protease inhibitors were included in all experimental steps and that the VP4-specific monoclonal antibody recognised only the intact VP4 (Fig. 5), yet the binding site is located on the VP5 cleavage fragment [Nilsson and Svensson, 1995].

The immune responses to nonstructural proteins were modest and restricted to NSP2, an equivalent to NSP2 of group A rotavirus [Bremont et al., 1993] and previously shown to be immunogenic in humans [Svensson et al., 1987; Richardson et al., 1993; Colomina et al., 1998]. That the immune responses to nonstructural proteins were low or absent in several individuals may suggest low immunogenicity and a limited exposure to the human immune system. This could be caused by either limited replication in the host or that the number of reinfections are few as compared with group A infections, in which the immune responses to nonstructural proteins are significant [Svensson et al., 1987; Richardson et al., 1993] and reinfections occur throughout life.

Whereas a significant amount information concerning the immune responses to group A rotaviruses has been accumulated during the years, the corresponding information regarding group C rotavirus infections is limited. Tsunemitsu et al. [1992b] proposed the existence of two distinct serotypes, and possibly a third, among human and animal viruses. We found that 16/19 group C antibody-positive individuals had antibodies that could neutralise heterologous group C rotavirus, suggesting that VP7 and/or VP4 from human and porcine group C strains share critical epitopes involved in neutralisation. Whether both outer capsid proteins (VP4 and VP7) of group C rotavirus are able to stimulate homologous or heterologous neutralising antibodies is not known, but at least VP4 can stimulate the formation of homologous neutralising antibodies [Nilsson and Svensson, 1995].

Our serological data suggest that group C rotavirus infections are more common in Sweden than detection rates have indicated previously [Svensson et al., 1986], possibly due to investigation of the wrong age groups.

It is suggested, based on available data, that group C rotavirus is primarily a clinical pathogen in older children and adults in Sweden and the Nordic countries, and thus has an epidemiological pattern different from group A rotavirus. In fact, none of the group C rotavirus detected previously in Sweden and Finland were isolated from children younger than 4 years [von Bonsdorff and Svensson, 1988; Maunula et al., 1992; Nilsson et al., unpublished]. We also believe that group C rotaviruses would be found more frequently in the stools of older children and adults with acute diarrhoea if adequate methods were used.

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